Foraging Behavior of *Anopheles gambiae* (Diptera: Culicidae) on *Parthenium hysterophorus* (Compositae) and *Senna occidentalis* (Fabaceae)

Honors Research Thesis

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by

Julie Guseman

The Ohio State University

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Project Advisor: Professor Woodbridge A. Foster
Department of Evolution, Ecology & Organismal Biology

Abstract

Foraging behavior of *Anopheles gambiae*, a malaria vector, was analyzed on *Parthenium hysterophorus* and *Senna occidentalis* through the use of video recordings. The two plant species are invasive plants known to be exceptionally attractive to *An. gambiae* but thought to yield very different amounts of sugar. With the importance of sugar as an energy reserve for flying, mating, blood feeding, and reproduction, foraging and finding access to sugar is a critical component of the life of a mosquito. Due to the differences in sugar production of the two plants, I hypothesized that foraging behavior of *An. gambiae* would differ greatly between the two plants, with more sugar being obtained from *S. occidentalis* On *P. hysterophorus*, the putative sugar-poor plant host, mosquitoes spent significantly more time on plants with flowers than without. On plants with flowers, both males and females exhibited a directed search for sugar, landing and foraging primarily on the prominent flowers and spending well over half of their time involved in foraging behavior (probing) on all parts of the plant. Males and females spent 80% and 58% of their time, respectively, on flowers, with 99% and 77% of that time devoted to foraging. However, for all their effort, they seldom obtained a sugar meal, with only 3.1% of males and 11.3% of females testing positive for fructose. On *S. occidentalis*, which produces visible amounts of sugar on prominent extra-floral nectaries, males and females spent 22% and 32% of their time, respectively, on EFNs, with 86% and 88% of that time devoted to foraging. After foraging on *S. occidentalis*, 46.2% of males and 50% of females were positive for fructose. *S. occidentalis* was more beneficial to *An. gambiae* in terms of energetics, and should be the focus of further malaria-control research.

Introduction

Anopheles gambiae Giles mosquitoes are significant malaria vectors in Sub-Saharan Africa, and as such, understanding their behavior is key to learning how to better control their populations and the transmission of malaria. Although female mosquitoes require blood for reproduction, their survival and fecundity are higher when given access to sugar, and males rely exclusively on sugar for energy (Gary and Foster 2001, 2006; Straif and Beier 1996). Sugar feeding in females and males almost always first occurs within 1-2 days after emergence, with females often taking sugar before blood (males do not bloodfeed) (Foster 1995). However, the presence of a blood host significantly increases the female's chance of first taking a blood meal, although smaller females are more likely to take sugar and then blood in the same night (Stone et al. 2012). Additionally, infection with the malaria parasite, *Plasmodium falciparum,* increases the female's attraction to plant odors and stimulates foraging behavior on plants (Nyasembe et al. 2014). With the importance of sugar as an energy reserve for flying, mating, blood feeding, and reproduction, foraging to find access to sugar is a critical component of the life of a mosquito.

Mosquitoes obtain sugar primarily from floral nectaries (flowers), and less often from extra-floral nectaries, honeydew, tree sap, and rotting fruit (Foster 1995). Plant piercing by mosquitoes has also been documented (Müller and Schlein 2005). Certain plants are known to be more attractive to *An. gambiae* than others (Manda et al. 2007). One of these plants is *Parthenium hysterophorus* L.*,* which produces small amounts of sugar in floral nectaries. *P. hysterophorus* has been identified as a primary nectar source for other insects, including bees (Bhusari et al. 2010); however, it was shown that even with its high level of attractiveness, survival and fecundity of *An. gambiae* on *P. hysterophorus* were comparable to negative controls, i.e., water (Manda et al. 2007). *Senna occidentalis*, another plant found to be attractive

to *An. gambiae*, produces visible amounts of sugar primarily through extra-floral nectaries, which are found at the intersections of petioles and stems. Due to these differences in sugar availability, I hypothesized that foraging behavior of *An. gambiae* would differ greatly between the two plants, with more sugar being obtained from *S. occidentalis*. It is the goal of this project to analyze the foraging behavior of *An. gambiae* mosquitoes on *P. hysterophorus* and *S. occidentalis*, through the use of infrared video recordings, to answer the following questions:

- Do *An. gambiae* mosquitoes exhibit a systematic search for sugar?
- Do *An. gambiae* show a preference for sugar-producing structures (flowers and extra-floral nectaries) over stems and leaves?
- Do foraging behaviors of *An. gambiae* result in a sugar meal?
- Do foraging patterns of *An. gambiae* differ between *P. hysterophorus* and *S. occidentalis*?

Methods

Mosquito rearing and maintenance

An. gambiae used in these experiments were of the Mbita strain, originally colonized and identified from specimens collected in 2001at Mbita Point, Suba District, Nyanza, Kenya by the staff of the International Centre of Insect Physiology and Ecology (ICIPE) . A colony of this strain, maintained at The Ohio State University (Biosafety protocol No. 2005R0020), provided the eggs used in the present study.

Mosquitoes were reared in an environment with ambient colony conditions of 27 ± 1°C, 80 ± 5% RH, and LD 13:11 h. Larvae were reared according to the methods of Gary and Foster (2006), except the experimental rearing procedure was modified to 300 larvae per pan in order to

produce smaller mosquitoes, which have smaller initial energy stores and therefore are more likely to feed shortly after emergence (Stone et al. 2012). Pupae were collected and placed in 30 x 30 x 50-cm clear acrylic cages to emerge. Newly emerged adults (<1d) were given access to water on cotton wicks before being transferred to experimental cages.

Experimental Design

Two recording setups were established. Each was comprised of two dome-shaped 67 x 67 x 67-cm insect cage composed of clear plastic sheeting on the sides and white plastic sheeting on the floor (MegaView Science Co., Ltd). Each cage had a zippered opening in the front, including a smaller 19-cm diameter netting-sleeved entrance that provided additional access to the cage. A single X-shaped slit was cut into the bottom of each cage through which a portion of a live plant would be inserted. In addition to the plant, mosquitoes were given access to a water wick and to a black plastic cup with 10 cm diameter to be used as a resting site.

One infrared video camera (Ikegami) was placed through the sleeve in the front of each cage, with the fabric netting secured around the cords of the camera to prevent the mosquitoes from escaping. Each infrared video camera was connected to a computer with video recording software. Initial videos were taken using exacqVision, while later recordings were made with NOLDUS Media Recorder. Two 940-nm infrared lights were directed at each cage, one above and one in the front, because infrared light above 900 nm does not alter a mosquito's visual environment (Gibson 1995). Each cage also contained a HOBO to record temperature and relative humidity throughout the recording. Wet cotton batting covered the floor of the cage to help maintain humidity. The setup was located in a climate-controlled room with lights on timers and rheostats to simulate dusk (19:30-20:00) and dawn (7:30-8:00). Recording took place over

night because *An. gambiae* is nocturnal. Total darkness was 20:00-7:30, and the recording time frame was 20:00-8:00. Temperatures averaged 25.4°C (S.D. = 0.70), with relative humidity averaging 75.5% (S.D. = 8.26).

Twenty to twenty-five males or females were released into each cage. The sexes were separated because video clarity did not allow differentiation between males and females. Mosquitoes were newly emerged (<1 day old) and had no previous access to sugar or plants. Mosquitoes were released into cages at least 3 hr before dusk to allow them to acclimate to the new environmental conditions. The following morning, mosquitoes were removed from the cage within one hour after the simulated dawn and were immediately transferred to a -70°C freezer, where they were kept until being tested for fructose content.

Treatments

Groups of male and female *An. gambiae* were exposed to four different treatments.

1. Treatment 1 used flowering *P. hysterophorus* plants. Four replicates established.
2. Treatment 2 used *P. hysterophorus* plants that had not yet flowered. Three replicates were performed.
3. Treatment 3 used unwashed non-flowering *S. occidentalis* with extra-floral nectaries. Three replicates were performed.
4. Treatment 4 used washed non-flowering *S. occidentalis* with extra-floral nectaries. In this treatment, plants were washed with water to remove excess sugar from the extra-floral nectaries in order to provide a comparison similar to treatment 2 (removal of sugar producing structures was not possible due to the nature of the plant) . Four replicates were performed.

Data Analysis

Video recordings were analyzed using NOLDUS The Observer XT software. Data on the number of mosquitoes on the plant were taken every 10 min. In addition, the behavior of single mosquitoes in Treatments 1 and 3 was recorded for 48 events each (24 male and 24 female) for the duration of their time on the plant. Four distinct behaviors (walking, walking/probing, probing, and resting) were recorded for three locations on the plant (stems, leaves, and flowers or extra-floral nectaries). Flying was also recorded when the mosquito did not leave the frame and returned to the plant immediately. Mosquitoes were also tested for the presence of fructose using the cold anthrone method of Van Handel (1972).

Using the JMP statistical package, a one-way analysis of variance was performed to determine the relationship between treatments in terms of number of mosquitoes on the plant, as well as the relationship between their initial landing site. A Pearson's goodness-of-fit test was performed to detect a relationship between initial landing sites and the efficiency of foraging strategy.

Results

Parthenium hysterophorus

The percentage of *An. gambiae* males and females on flowering (treatment 1) and non-flowering (treatment 2) of *P. hysterophorus* is shown in Figure 1. Males on plants with flowers were observed to have a spike of activity immediately after dusk, and they maintained a higher level of activity than females throughout the night. Females on plants with flowers were also active at relatively constant levels throughout the night. Activity of males and females was markedly lower on non-flowering *P. hysterophorus* plants as compared to these plants without

flowers, and all categories (as shown in Figure 1) were statistically significant (ANOVA, F = 263.5, $P<0.05$).

In both treatments, very few mosquitoes tested positive for sugar (Table 1). After exposure to flowering *P. hysterophorus*, 3.1% of males and 11.3% of females tested positive for fructose. After exposure to non-flowering *P. hysterophorus*, fructose was present in 0% of males and 3.6% of females.

48 events, 24 males and 24 females, were chosen at random from treatment 1, and their behaviors while on *Parthenium hysterophorus* were recorded and analyzed. The initial landing sites on the plant are shown in Figure 2. Both males and females landed on flowers more frequently (ANOVA, $P<0.05$) than on stems or leaves, which were not significantly different (ANOVA, $P>0.05$). Relative sizes of the three plant parts were calculated from the observable profile of the plant in the video frame, using the program Analyzing Digital Images. Although flowers made up 65-70% of initial landing events, in the video's observable frame, flowers accounted for only 40.5% of the plant's available surface, while stems made up 21.3% and leaves accounted for 38.2%. These differences between available surface area and initial landing site were statistically significant from the expected values (X^2, $p<0.05$). Additionally, after flight events observed during each event, mosquitoes landed on flowers much more often than on other parts of the plant (Figure 3, X^2, $p<0.05$).

Figure 4 shows the time spent by males (n=24) and females (n=24) on the three different parts of the flowering plant, and their behaviors when in those locations. Both males and females spent more than half of their time on flowers. While on flowers, males divided their time equally between probing, walking and probing, and resting, whereas females spent half of their time resting and the other half probing or walking and probing. On stems, males and females spent the

majority of the time resting. However, activity on leaves varied between males and females. While males devoted 82% of their time on leaves to probing behaviors (probing and walking/probing) and 13% to resting, females again rested for the majority of the time (65%). In total, females spent an average of 1928 seconds on the plant (S.D. = 2351 s), while males averaged 622.8 s (S.D. = 734.9 s).

During analysis of the behavior of males and females on all parts of the plant, it became evident that in almost all cases, one or two individuals accounted for the majority of the resting behavior observed, skewing the data in favor of high resting percentages. These individuals were removed from the data set, and the modified results are shown in Figure 5. Hence, two mosquitoes were removed from stems and flowers for both males and females (n = 22), and one female mosquito was removed from leaves (n = 23). Again, in this reduced sample, the greatest amount of time was spent on flowers, and this bias was especially strong in males. Probing behaviors (both probing and walking/probing) accounted for the majority of activity for both males and females on all parts of the plant. This was highest when males were on flowers; 99% of their activity was probing behaviors. On stems and leaves, males devoted 73% and 82% of their time, respectively, to probing and walking/probing. For females, probing behaviors were most common on leaves (92%), while it accounted for 77% of their time on flowers and 66% of their time on stems.

Of the 24 males and 24 females analyzed, the full behavior analysis of three representative males and three representative females can be seen in Figure 6. Individuals were deliberately chosen to show an array of behavioral patterns observed. Females 1 and 2 represent individuals who where fairly stagnant on the plant. These two individuals reflected eight total observations, and were characterized by long periods of time in one location. Female 3, Male 1

and Male 2 characterized mosquitoes that were very active on the plant; 35 total events fell into this category. Male 3 displayed behavior falling somewhere between the two previously mentioned groups. Seven events were in this category.

Efficiency of foraging behavior was assessed by recording visits of individual mosquitoes to new flowers as compared to previously visited flowers. Results are shown in Figure 7. On *P. hysterophorus*, 79% of males and 67% of females foraged on flowers. Both sexes showed no differentiation between new flowers and flowers on which they had previously foraged (X^2 = .23, P<0.05).

Senna occidentalis

Activity of males and females on both treatments with *Senna occidentalis* is shown in Figure 8. Males were more active than females on the plant in both treatments (ANOVA, F = 110.1, P<0.05), although there was no significant difference in the activity of males between treatments 3 and 4. There was a significant difference between females in treatments 3 and 4 (ANOVA, P<0.05). All four groups exhibited a general trend of increasing activity on the plant throughout the night. Unwashed plants providid sugar meals for 46.2% of males and 50% of females, while washed plants provided sugar meals for 39.5% of males and 29.8% of females (Table 2).

A random sample of 24 males and 24 females from treatment 3 was selected for individual behavioral analyses. Initial landing sites are shown in Figure 9. All three locations were significantly different from one another, with stems being most common (ANOVA, P<0.05). Initial landing site was not a function of the relative size of plant parts for males (X^2 = 604.5, P<0.05) or females (X^2 = 888, P<0.05). Stems accounted for 14.8% of the plant, while

46% of males and females landed there initially. Leaves were the largest portion of the plant (84.7%), yet only 37% of males and 33% of females landed there. Extra-floral nectaries comprised the smallest portion of the plant (0.5%), although 17% of males and 21% of females initially landed there.

Time distributions of males and females are shown in Figure 10. Both males and females divided their time fairly equally between the three parts of the plant (extra-floral nectaries, stems, and leaves). Although extra-floral nectaries accounted for only 22% of the time spent on the plant for males and 36% of time spent for females, foraging behaviors on extra-floral nectaries accounted for 86 and 88% of this time, respectively. Resting accounted for a much larger portion of behaviors on stems and leaves (37-51%). Individuals that spent a significant amount of time resting were removed, with the adjusted time budgets being shown in Figure 11. No females were removed as there were no females observed to spend an unusual amount of time resting. Two males were removed from leaves and one male was removed from stems. With these adjustments, males spent a majority of their time foraging on all plant parts, although the most foraging was while on extra-floral nectaries. Overall, females averaged 2926.2 s (S.D. = 2,043.1 s) on the plant, with males averaging 5939.47 s (S.D. = 7,257.91 s).

Analysis of efficiency of foraging revealed that on *S. occidentalis*, 46% of males and 33% of females from the individual behavioral analyses foraged on extra-floral nectaries. Of those that did not land directly on extra-floral nectaries, all transitions to extra-floral nectaries occurred from stems. Individual linear time budgets for three males and three females are shown in Figure 12 and display representative samples of individual behavior. Female 1, Female 2, and Male 1 exhibit stem-to-EFN transitions and foraging. Female 3 and Male 3 represent those

individuals that did not forage on extra-floral nectaries. Male 3 is representative of individuals that initially foraged on extra-floral nectaries.

Discussion

The results confirm the findings of previous studies (Manda et al. 2007) in regards to the attractiveness of *P. hysterophorus* and *S. occidentalis*. *P. hysterophorus* (treatment 1) was more attractive than *S. occidentalis* (treatment 3), with more individuals observed on *P. hysterophorus* throughout the night (ANOVA, $P<0.05$). The results indicate that females from both treatments were less attracted to their respective plants than males of the same treatments, although they are more likely to obtain sugar meals than males. Additionally, the presence of sugar-positive females from treatment 4 could provide additional support for the findings of Müller and Schlein (2005), that female *An. sergentii* mosquitoes were able to pierce plants to obtain sugar in addition to probing sugar-producing structures.

Initial landing site was not a function of the relative size of plant parts in either plant; therefore, attraction must be due to some other factor. Work has been done to determine the volatiles produced by *P. hysterophorous* that are attractive to *An. gambiae* (Nyasembe et al. 2012). Flowers were by far the most attractive part of *P. hysterophorus*. The most attractive part of *S. occidentalis* was not the sugar producing structure; however, the fact that extra-floral nectaries make up such a small portion of the plant and are located on stems could account for stems being observed as the most frequent initial landing site. On both plants, males and females showed the same preferences for initial landing sites, indicating that the factors that cause attractiveness are perceived similarly by both sexes.

Differences in foraging patterns were observed between mosquitoes foraging on the two plants. On *P. hysterophorus*, individuals landed most commonly on flowers and spent most of their time foraging on flowers. However, foraging was often inefficient, and individuals were unable to identify flowers on which they had previously foraged. Individuals spent less time foraging on *P. hysterophorus* than on *S. occidentalis*, which could indicate that they are able to identify some threshold at which foraging without finding sugar is no longer beneficial. On *S. occidentalis*, fewer individuals were able to locate sugar producing structures, and time was divided equally between all three plant parts. Foraging was less directed than on *P. hysterophorus*, which could be due to the lower attractiveness observed on *S. occidentalis*. However, individuals foraged for longer periods of time on *S. occidentalis*, indicating that individuals may be able to identify some cue to the presence of sugar. *An. gambiae* mosquitoes do recognize their presence on a plant as a cue to engage in foraging behaviors, as probing was the most common behavior observed on all plant parts (excluding resting outliers).

Smith and Gadawski's study of the foraging behavior of *Aedes provocans* (1994) was a point of reference for this study, as it is the only previous study of specific foraging behaviors in mosquitoes on plants. General trends in foraging behavior were similar between the two studies. Males and females engaged in behaviors at similar frequencies, although wide ranges of behaviors were observed across all individuals. Both studies also found an inefficiency to foraging strategy, with males and females frequently returning to the same sugar-producing structures. It is not clear whether this is due to an inability to recognize previous foraging locations, or if it is possible that they are returning to sugar-producing structures that still contain available sugar. While these similarities are promising, the lack of published data on the foraging

behavior of mosquitoes makes it difficult to indicate whether these patterns are true across all mosquitoes.

Although *P. hysterophorus* is more attractive, mosquitoes spend more time foraging on *S. occidentalis* and are more likely to obtain a sugar meal as a result. *P. hysterophorus* is often targeted as a subject of study in malaria-control research due to its attractiveness and possible presence of ovicidal or antimalarial chemicals (Kumar et al. 2011), yet it does not appear to be a beneficial plant to *An. gambiae* in terms of energetics. Further studies involving additional plant species could determine energetic benefits and associations between *An. gambiae* and local flora. While *P. hysterophorus* may be beneficial chemically, elimination and management of *S. occidentalis* as a sugar source in residential areas of Sub-Saharan Africa could help reduce *An. gambiae* populations and their impact on local people.

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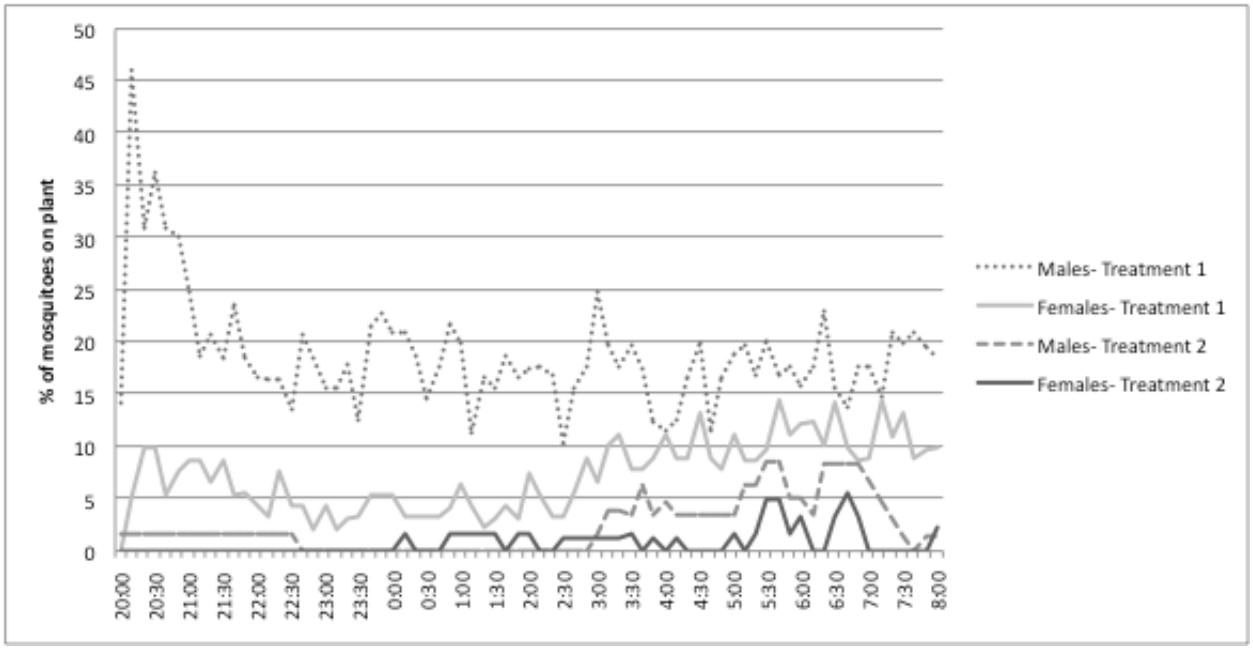


Figure 1: Percentage of male and female *An. gambiae* on *P. hysterophorus* plants with flowers (treatment 1) and without flowers (treatment 2). n=98 (Males, treatment 1); 97 (females, treatment 1); 56 (males, treatment 2); 56 (females, treatment 2). All treatments were significantly different from each other (ANOVA, $P<0.05$).

Table 1: Results of cold anthrone testing of *An. gambiae* males and females on *P. hysterophorus* with flowers (treatment 1) and without flowers (treatment 2)

	Presence of Fructose*
Male- Treatment 1 (n=98)	3.1%
Female- Treatment 1 (n=97)	11.3%
Male- Treatment 2 (n=56)	0%
Female- Treatment 2 (n=56)	3.6%

*As indicated by a positive cold anthrone test

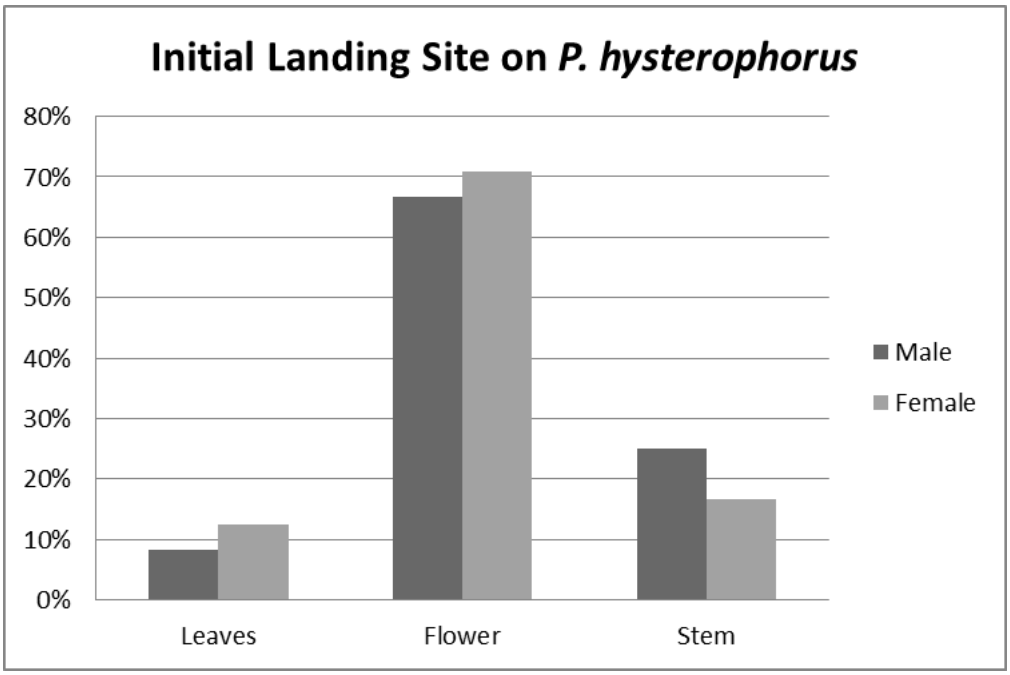


Figure 2: Initial landing site of male (n=24) and female (n=24) *An. gambiae* on *P. hysterophorus*. Flowers were significantly different from both leaves and stems (ANOVA, $p<0.05$).

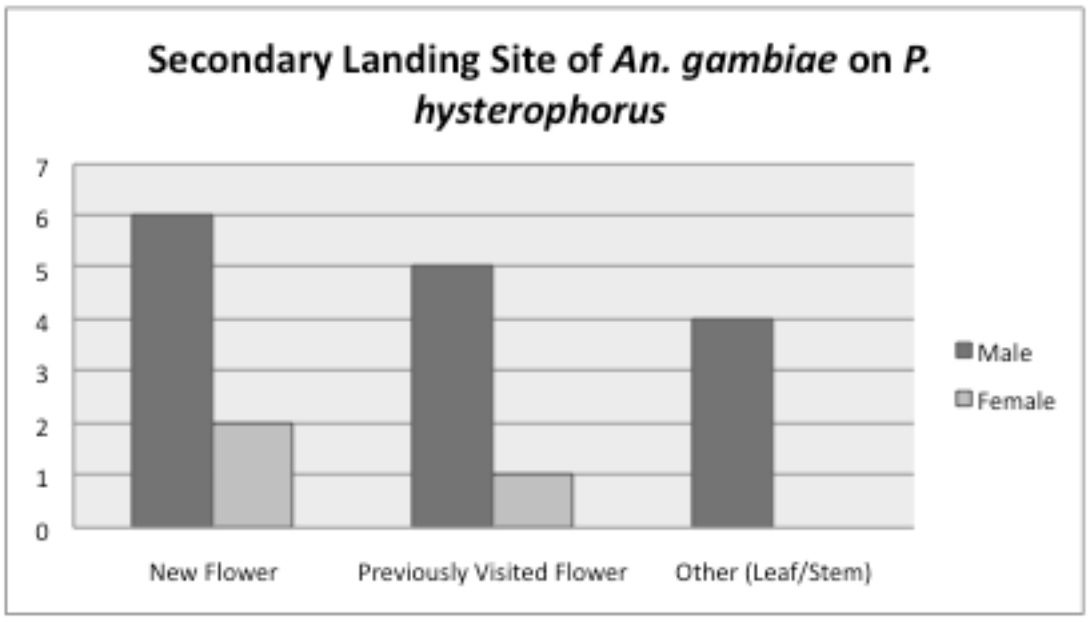


Figure 3: Foraging location of male and female *An. gambiae* after flight. Of the 19 males and 16 females observed exhibiting foraging behavior on flowers, one female and four males also exhibited flying behavior during their foraging. These secondary landing sites were recorded as new flowers, flowers on which they had previously foraged, and other locations (leaves and stems). Flowers were visited significantly more often than leaves or stems (X^2, $p<0.05$).

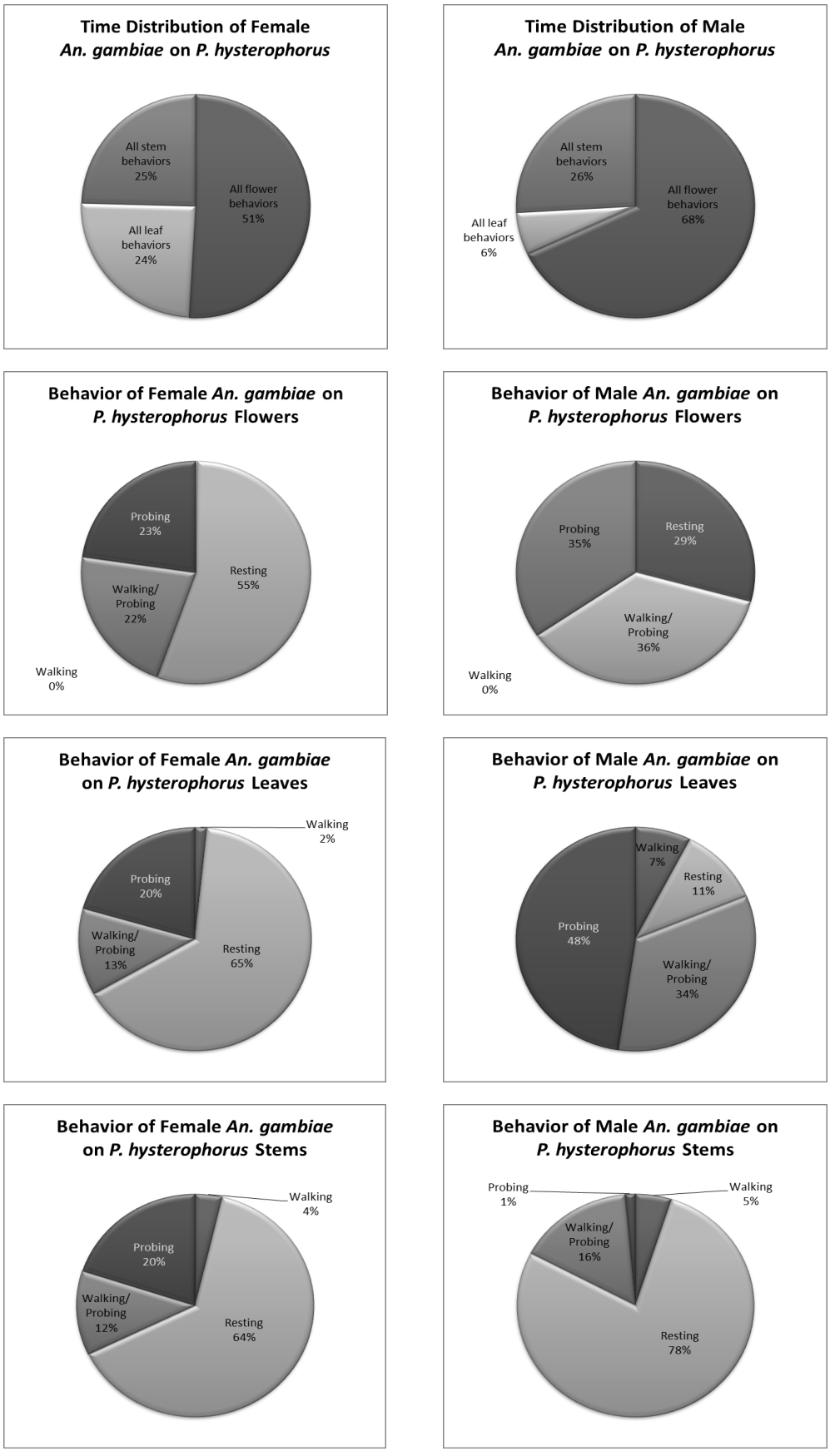
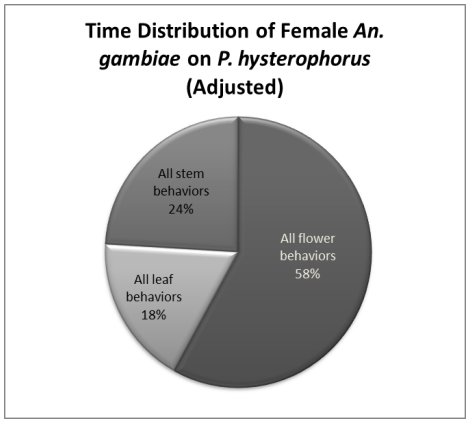
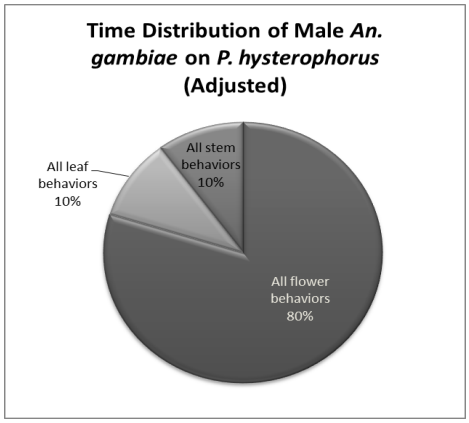
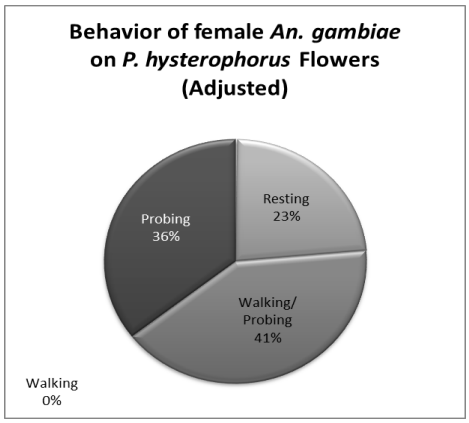
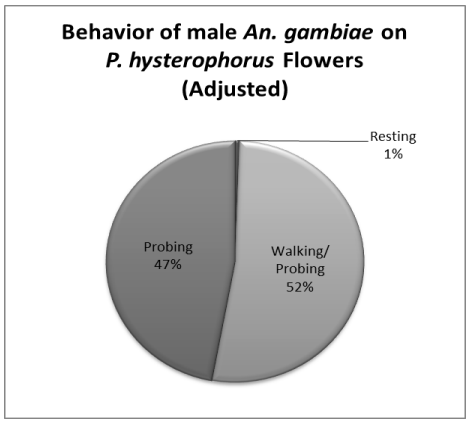


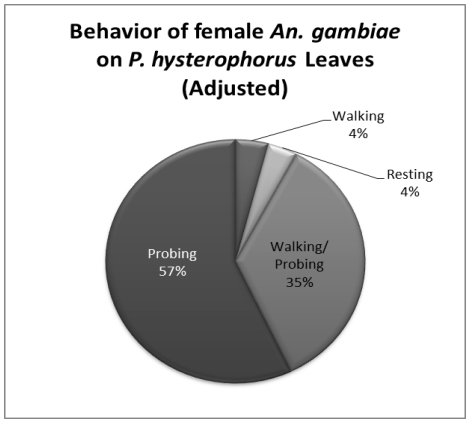
Figure 4: Behaviors of male (n=24) and female (n=24) *An. gambiae* on flowering *P. hysterophorus*

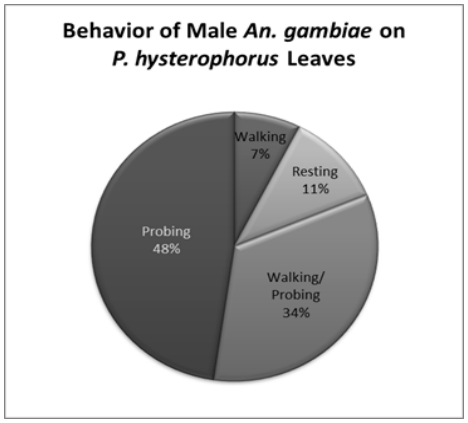


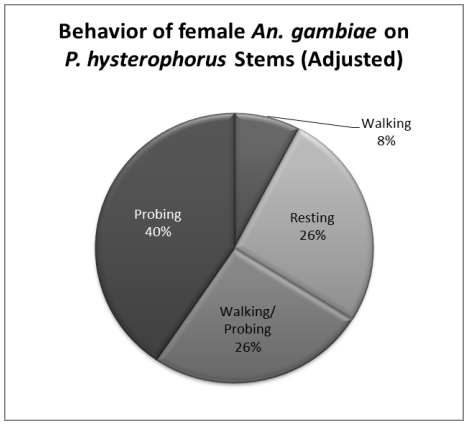












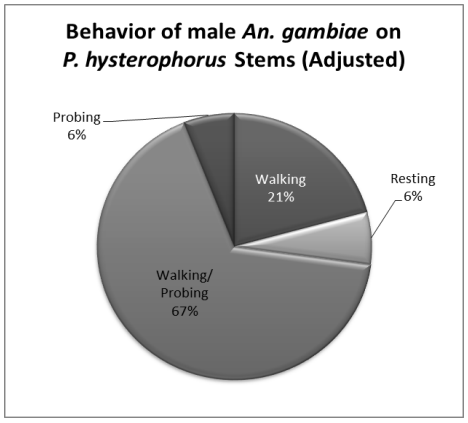


Figure 5: Time distribution graphs excluding mosquitoes that spent an unusual amount of time resting with little other activity. N=22 for stems and flowers (male and female); N=23 for leaves (female); N=24 for leaves (male). Excluded mosquitoes accounted for 79-99% of resting behavior observed on that part of the plant.

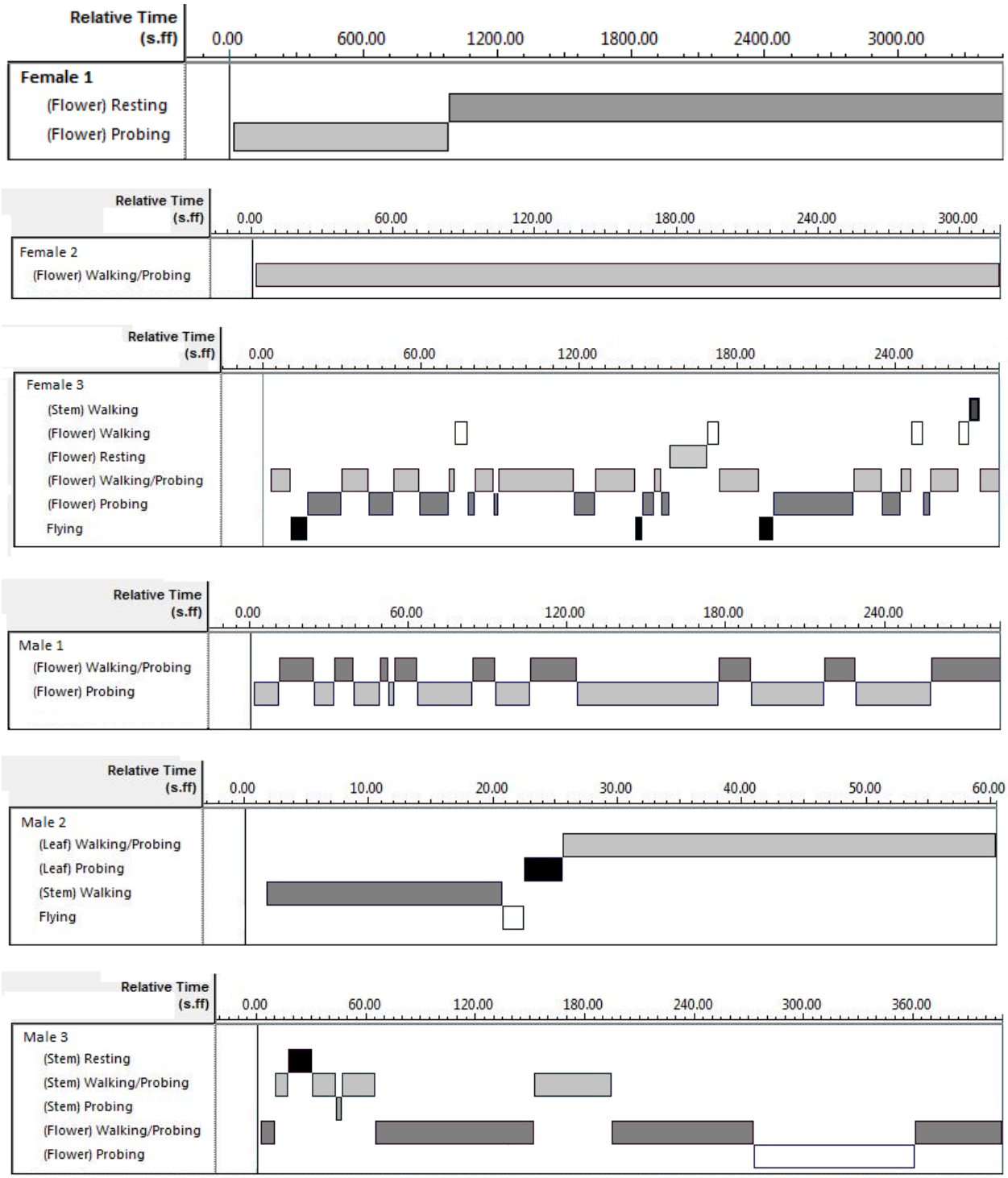


Figure 6: Behaviors of three representative males and three representative females on *Parthenium hysterophorus* (treatment 1), according to type of foraging pattern. Relative time is time elapsed in seconds.

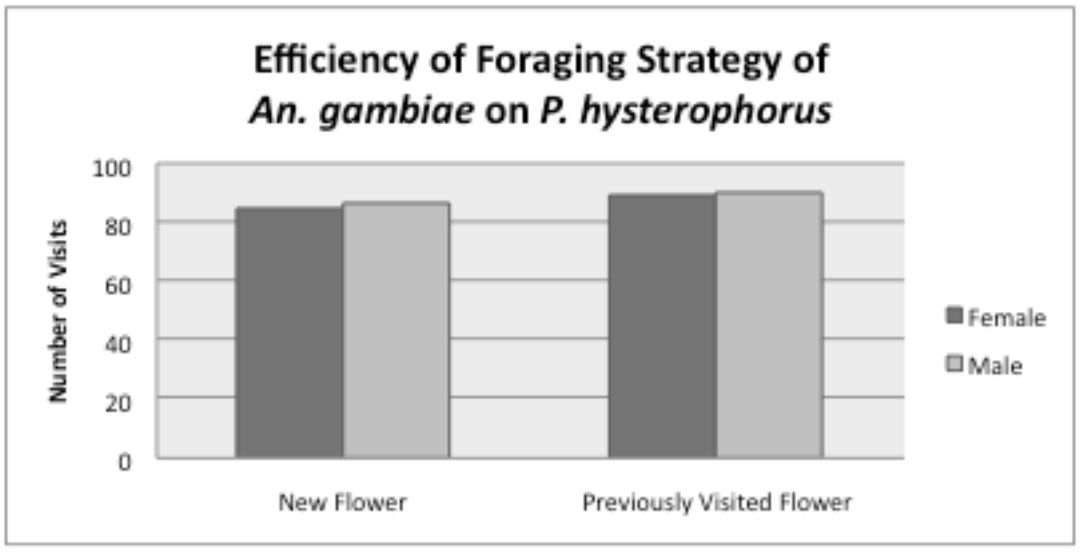


Figure 7: Efficiency of foraging strategy of male and female *An. gambiae.* Foraging behaviors (walking and walking/probing) on flowers were observed in 19 males and 16 females and visits to new flowers and flowers on which the individual already foraged were recorded. Males and females that did not forage on flowers were excluded. Differences between new and previously visited flowers were not significant (X^2, $p<0.05$).

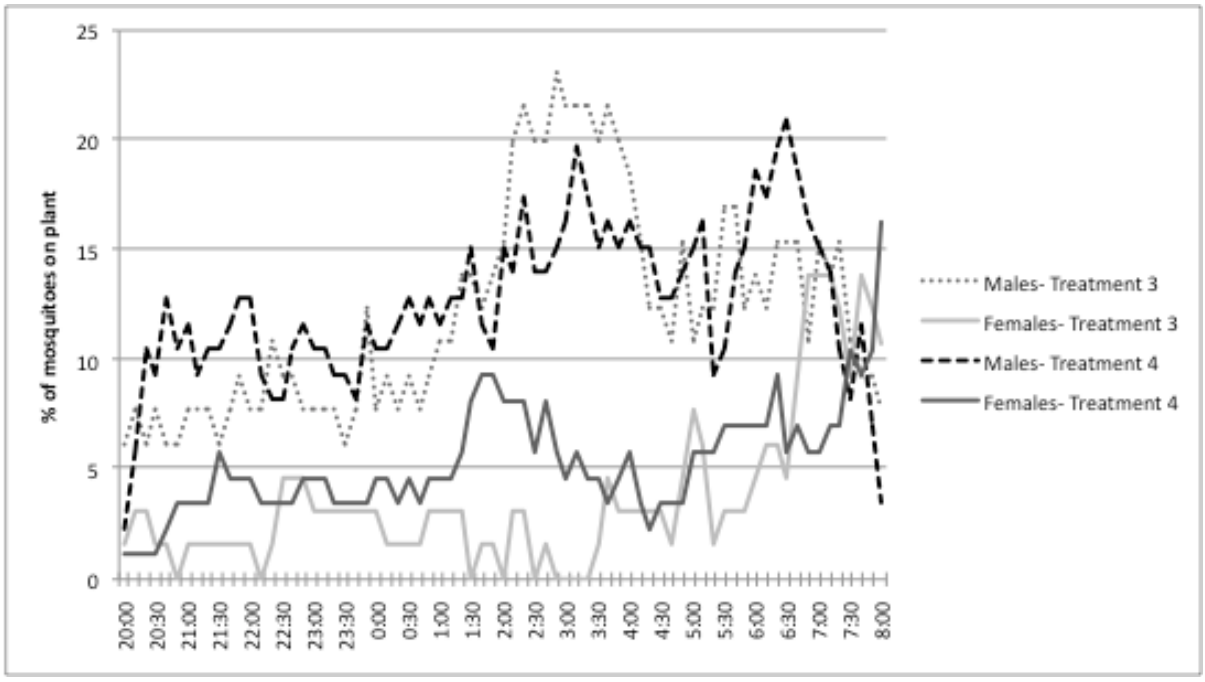


Figure 8: Activity of male and female *An. gambiae* on *S. occidentalis* plants, treatments 3 (unwashed *S. occidentalis)* and 4 (washed *S. occidentalis)*. n=65 (Males, treatment 3); 65 (females, treatment 3); 86 (males, treatment 4); 86 (females, treatment 4). Males in treatments 3 and 4 were not significantly different (ANOVA, $p>0.05$). Females in treatments 3 and 4 were significantly different from each other as well as from the two treatments with males(ANOVA, $p<0.05$).

Table 2: Results of cold anthrone testing of males and females on *S. occidentalis* washed (treatment 3) and unwashed (treatment 2).

	Positive*
Male- Treatment 3 (n=65)	46.2%
Female- Treatment 3 (n=62)	50.0%
Male- Treatment 4 (n=81)	39.5%
Female- Treatment 4 (n=84)	29.8%

*A positive test indicates presence of fructose, a plant sugar not synthesized by the insect.

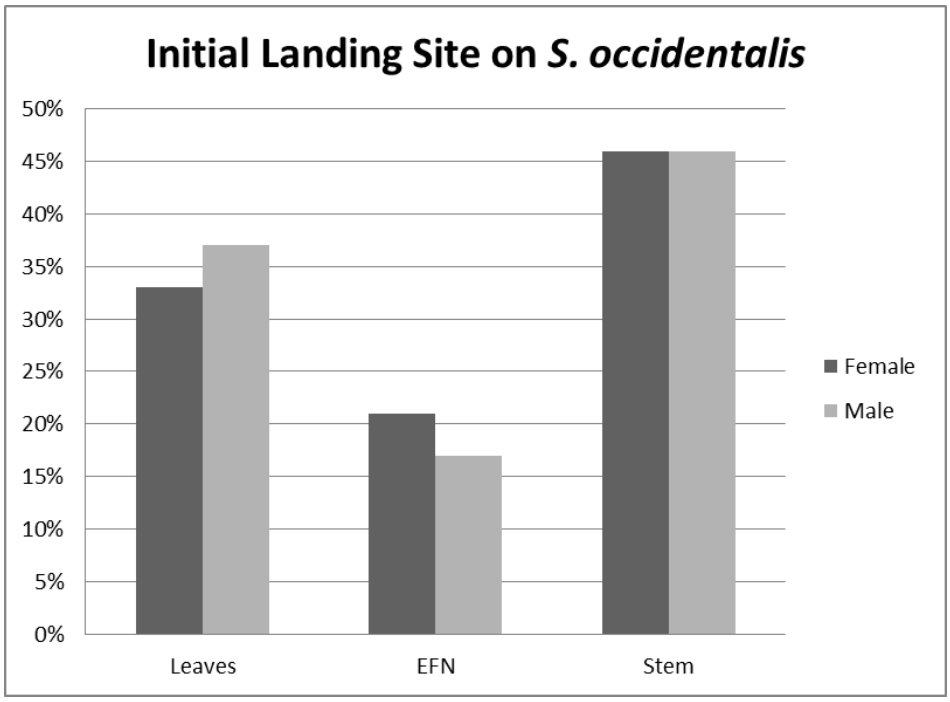


Figure 9: Initial landing site of male and female *An. gambiae* on *S. occidentalis*. The three locations (leaves, extra-floral nectaries, and stems) were all significantly different (ANOVA, $P<0.05$)

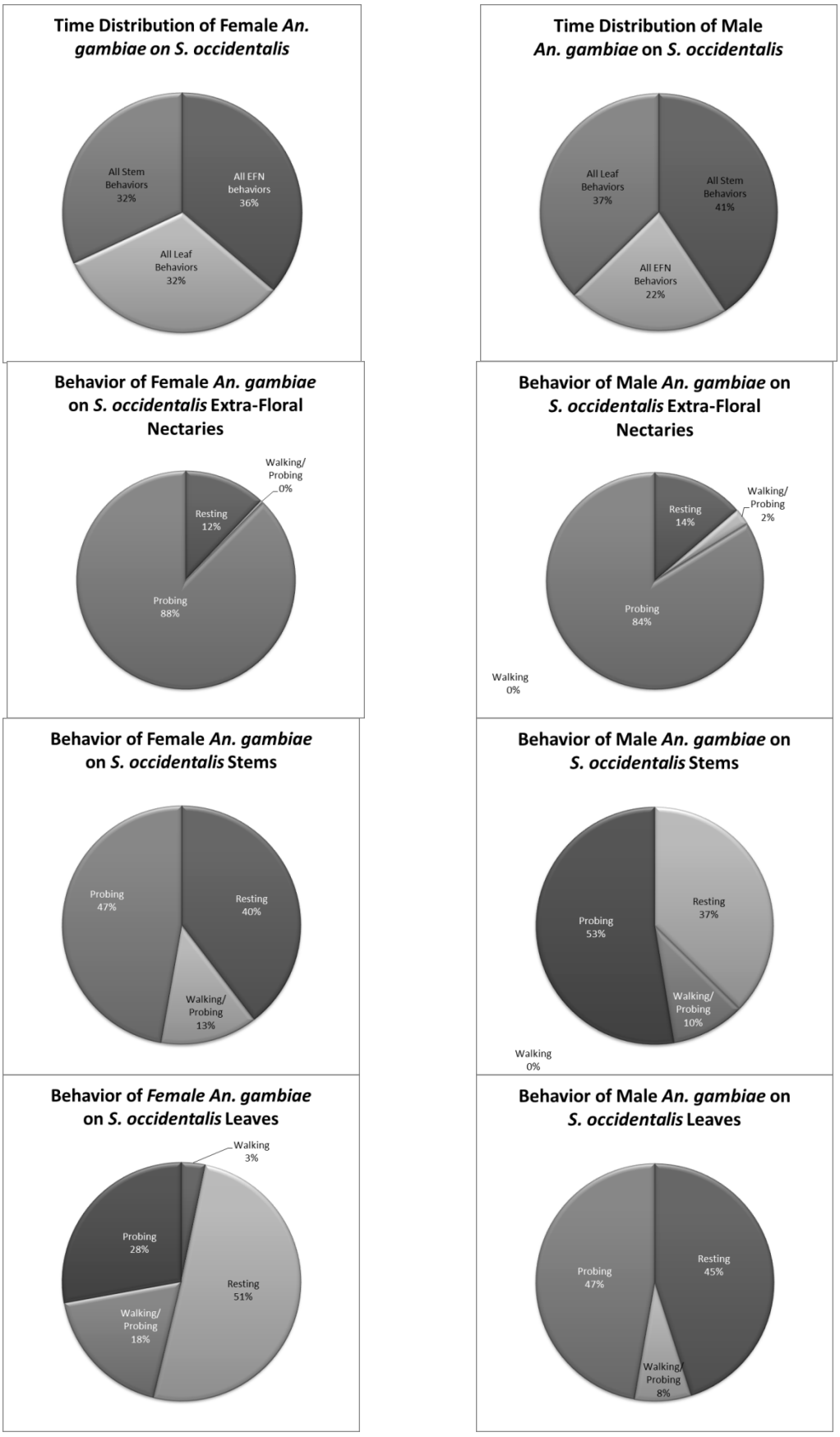


Figure 10: Behaviors of male (n=24) and female (n=24) *An. gambiae* on *S. occidentalis*

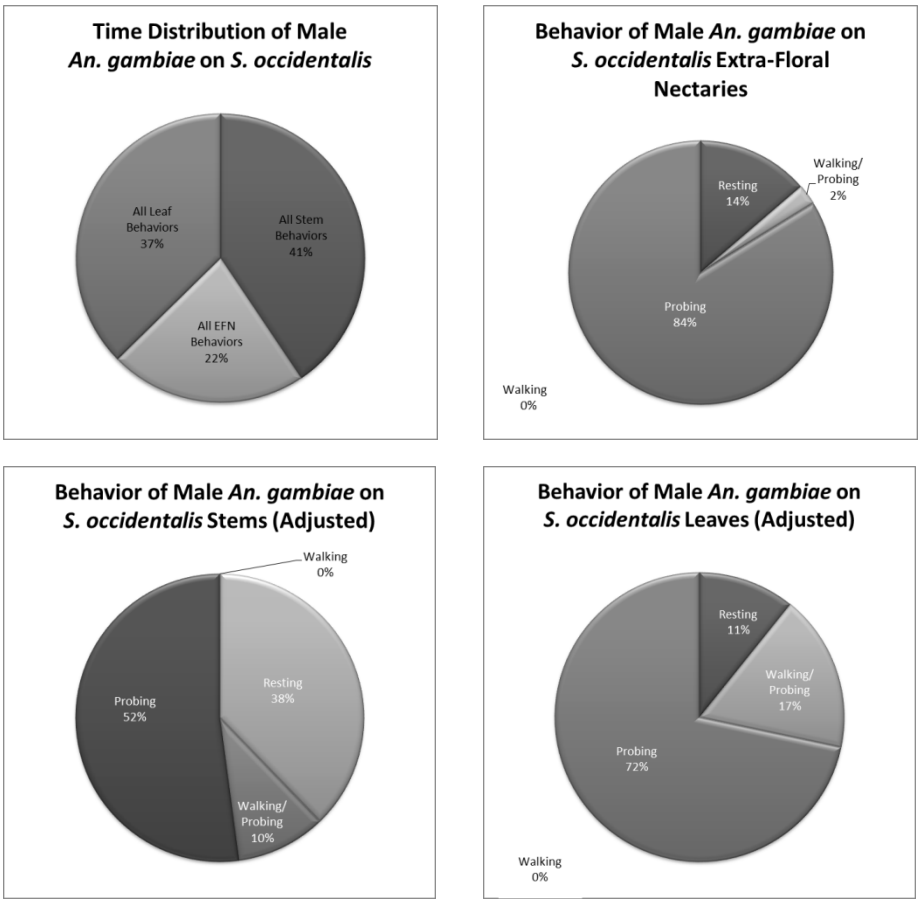


Figure 11: Time distribution graphs on *S. occidentalis* excluding mosquitoes that spent an unusual amount of time resting with little other activity. N=23 for stems (male); N=22 for leaves (male); N=24 for extra-floral nectaries (male). Excluded mosquitoes accounted for 84-90% of resting behavior observed on that part of the plant. No adjustments were made for females due to a lack of individuals qualifying for exclusion (unusual amount of time resting).

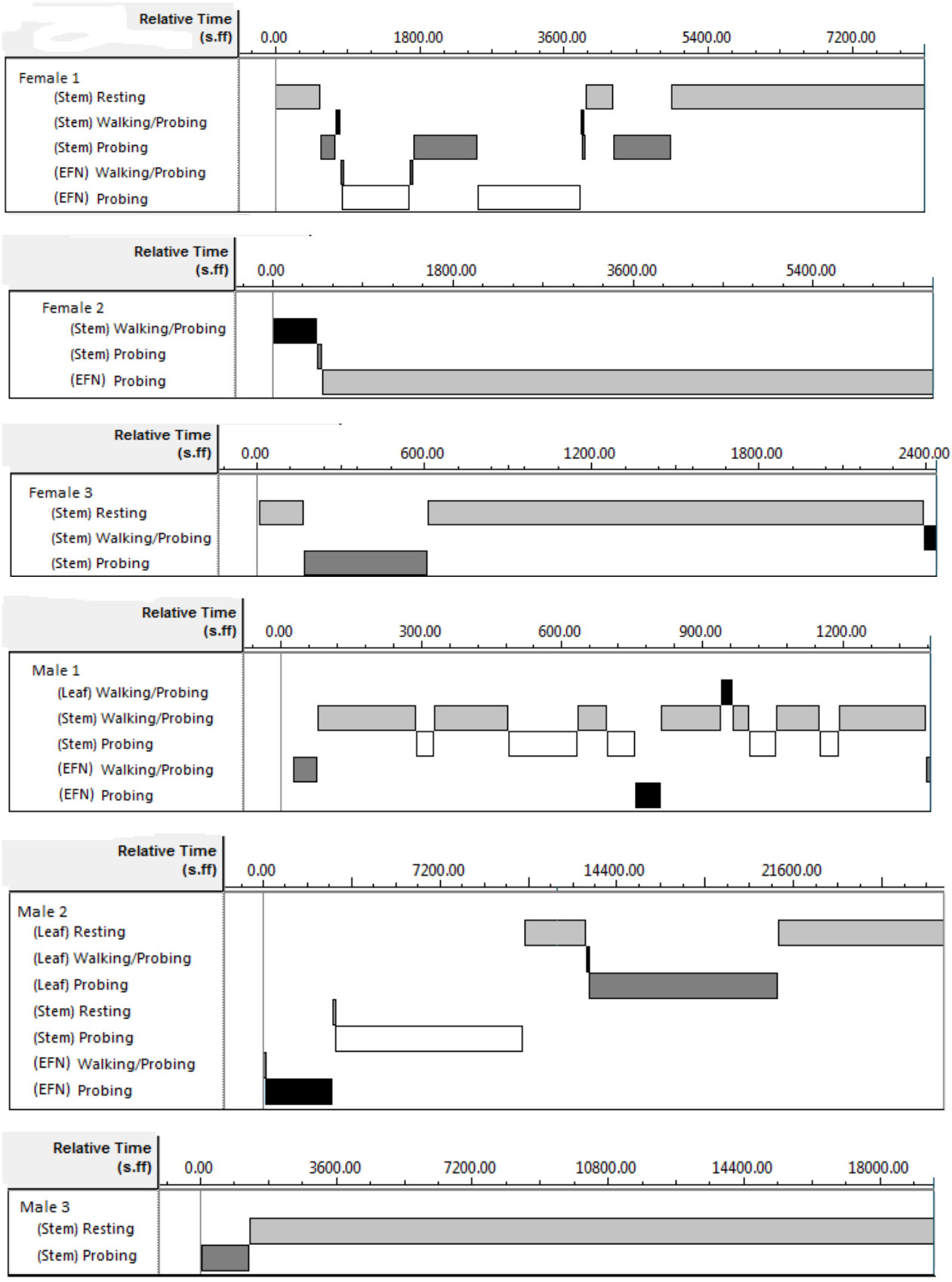


Figure 12: Behaviors of three representative females and three representative males on *Senna occidentalis* (Treatment 3), according to type of foraging pattern. Relative time is time elapsed in seconds.